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Angiotensin II inhibits uptake of transferrin-bound iron but not non-transferrin-bound iron by cultured astrocytes

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ABSTRACT

The existence of all components of the renin–angiotensin system (RAS) and the iron metabolism system, and the recent findings on the functions of angiotensin II (ANGII) in peripheral iron metabolism imply that ANGII might play a role in iron homeostasis by regulating expression of iron transport proteins in the brain. Here, we investigated effects of ANGII on uptake and release of iron as well as expression of cell iron transport proteins in cultured astrocytes. We demonstrated that ANGII could significantly inhibit transferrin-bound iron (Tf-Fe) uptake and iron release as well as the expression of transferrin receptor 1 (TfR1) and the iron exporter ferroportin 1 (Fpn1) in cultured astrocytes. This indicated that the inhibitory role of ANGII on Tf-Fe uptake and iron release is mediated by its negative effect on the expression of TfR1 and Fpn1. We also provided evidence that ANGII had no effect on divalent metal transporter 1 (DMT1) expression as well as non-transferrin-bound iron (NTBI) uptake in the cells. Our findings showed that ANGII has a role to affect expression of iron transport proteins in astrocytes in vitro and also suggested that ANGII might have a physiological function in brain iron homeostasis.

1. Introduction

The primary function of the renin–angiotensin system (RAS) is to maintain fluid homeostasis and regulate blood pressure (Savaskan, 2005). Not only does it function as an endocrine system, but it also serves local paracrine and autocrine functions in tissues and organs (Mehta and Griendling, 2007). Angiotensin II (ANGII) is the primary effector molecule of this system which has emerged as a critical hormone that affects the function of virtually all organs, including heart, kidney and also brain (Dinh et al., 2001; Mehta and Griendling, 2007). In some peripheral organs and cells, a number of studies demonstrated that ANGII is able to regulate the expression of iron metabolism proteins and hence affect iron homeostasis.

It has been reported that ANGII could induce a significant increase in the expression of transferrin receptor 1 (TfR1), divalent metal transporter 1 (DMT1), ferroportin 1 (Fpn1) and hepcidin in rat kidney (Ishizaka et al., 2007), cause iron accumulation and ferritin induction in rat aorta (Ishizaka et al., 2005a), kidney, heart and liver (Ishizaka et al., 2005b), and promote non-transferrin-bound iron (NTBI) uptake by bovine endothelial cells (Mak et al., 2012) and facilitated the expression of iron uptake and release proteins including TfR1, DMT1 and Fpn1 and increased the intracellular iron concentration as well as labile ferrous iron in human glomerular endothelial cells (Tajima et al., 2010). ANGII-induced deposition of iron has been considered to be at least partly associated with ANGII-induced renal or endothelial injury, impairment of vascular function, and arterial remodeling by the enhancement of oxidative stress (Ishizaka et al., 2002).

All the required components of the RAS, including ANGII and its type 1 (AT1) and 2 (AT2) receptors (Sumners et al., 1991; Rydzewski et al., 1992; Garrido-Gil et al., 2013b), renin, angiotensinogen and angiotensin-converting enzyme (ACE) have been demonstrated to be present in the mammalian brain (Lippoldt et al., 1995; Hadjiivanova and Georgiev, 1998; Inagami et al., 1999; Gasparo et al., 2000; Mayas et al., 2005). All key proteins involved in iron metabolism and transport, such as TfR1, DMT1, Fpn1 and iron regulatory peptide hepcidin, have been found to express in the brain as well (Qian and Wang, 1998; Qian and Shen, 2001; Ke et al., 2005; Zechel et al., 2006; Wang et al., 2008). The existence

Abbreviations: ACE, angiotensin-converting enzyme; AD, Alzheimer's disease; ANGII, angiotensin II; DMT1−IRE, divalent metal transporter 1 without iron response element; DMT1+IRE, divalent metal transporter 1 with iron response element; Fpn1, ferroportin 1; GFAP, glial fibrillary acidic protein; NTBI, non-transferrin bound iron; PBS, phosphate buffered saline; Tf-Fe, transferrin-bound iron; TfR1, transferrin receptor 1.

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of all components of the RAS and the iron metabolism systems, and the current understanding of ANGII functions in peripheral iron metabolism suggested strongly that ANGII might play a role to affect iron homeostasis by regulating expression of iron transport protein in the brain. A recent study by Garrido-Gil et al. (2013a) provided the first piece of convincing evidence for this hypothesis. They demonstrated that administration of angiotensin to primary mesencephalic cultures, the dopaminergic cell line MES23.5 and to young adult rats, significantly increased levels of TfR1, DMT1, and Fpn1 via type-1 receptors. In the present study, we investigated effects of ANGII on uptake and release of iron as well as expression of cell iron transport proteins in cultured astrocytes in vitro and found that ANGII inhibited uptake of transferrin-bound iron but not non-transferrin-bound iron in the cells.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA. The scintillation cocktail and tubes were purchased from Beckman Coulter Company, Fullerton, CA, USA and $^{55}FeCl_3$ from PerkinElmer Company, Wellesley, MA, USA. The mouse anti-rat transferrin receptor 1 monoclonal antibody was obtained from BD Transduction Laboratories, BD Biosciences Pharmingen, USA and antibodies against divalent metal transporter with (DMT1+IRE) or without iron response element (DMT1−IRE) and ferroportin 1 were purchased from Alpha Diagnostic International Company, San Antonio, TX, USA. The specific antibody against astrocyte glial fibrillary acidic protein (GFAP) was purchased from Chemicon International Ltd., UK. Male Sprague–Dawley (SD) rats were obtained from the animal center of the Third Military Medical University. The Animal Ethics Committees of the University approved the use of animals for this study.

2.2. Primary astrocytes

Primary astrocytes were prepared from 1-day-old SD rats by a procedure previously described (Qian et al., 2000; Du et al., 2011). Briefly, cerebral cortex was digested with 0.25% trypsin for 30 min at 37 °C. After trypsinization and trituration, cell suspensions were sieved through a 40-mm cell strainer and the filtrate was allowed pre-adherence for 30 min to remove any contamination from fibroblast. The plated cells were incubated in a 5% CO2 incubator (NAPCO 5400) at 37 °C. After the cultures reached confluence (12–14 days), they were subcultured 3 times at a 4-day interval and allowed pre-adherence for 30 min before being seeded in each subculture. The purity of the astrocyte cultures was assessed by staining for the astrocyte marker anti-GFAP antibodies, which was approximately 99%.

2.3. Measurement of transferrin-bound iron (Tf-Fe) uptake

^{55}Fe-Tf were prepared first by mixing ^{55}FeCl3 (Perkin–Elmer) with nitrilotriacetic acid (NTA) in a 1:10 ratio and then incubating ^{55}Fe-NTA with apo-Tf in a 2:1 ratio for 3-h in carbonate buffer (10 mM $NaHCO_3$, 250 mM Tris–HCl) (Qian and Morgan, 1990, 1991). After being incubated with serum-free culture medium containing 0.1% BSA at 37 °C for 1-h to remove any endogenous transferrin, astrocytes were treated with ANGII and then incubated with or without ^{55}Fe-Tf in 0.155 M NaCl buffered by pH 7.4 with 4 mM Hepes. After centrifugation to remove cell surface-bound radioactive ^{55}Fe-Tf, the cells were washed again with ice-cold phosphate buffered saline (PBS), lysed in 1% SDS, and counted for 10-min in a scintillation counter (Perkin–Elmer). The counts represented the ^{55}Fe taken up by the cells.

2.4. Measurement of non-transferrin bound iron uptake

The radio-labeled ^{55}Fe(II) (NTBI) solution was prepared and the Fe(II) uptake was measured as described previously (Qian et al., 1996, 2000). After being incubated with ANGII and ^{55}Fe(II) in 0. 27 M sucrose buffered by pH 6.5 with 4 mM Pipes, the cells were lysed, scraped off and transferred into Eppendorf tubes. A 50 μl aliquot was subjected to the detection of protein concentration. The cytosol was separated from the stromal fraction by centrifugation at 10,000*g* for 20-min at 4 °C using a Jouan centrifuge (DJB labcare Ltd., England). Scintillation solution (3 ml) was added to determine the counts per minute (cpm). The total iron uptake was the sum of the cytosol and stromal fractions.

2.5. Iron release assay

The astrocytes were incubated with ^{55}Fe(II) and then with ANGII. The medium was then collected and the cpm measured following centrifugation. The cells were detached by 500 μl lysis buffer. A 50-μl aliquot was subjected to the detection of the protein concentration. The cytosol was separated from the stromal fraction by centrifugation at 10,000*g* at 4 °C for 20-min. A 3 ml scintillation solution was added to both fractions to count the cpm. The sum of the radioactivity in the medium and in the cell (cytosol and stromal fractions) was named the total cellular radioactivity. The relative percentage of the total radioactivity in the medium and in the cell was calculated. The percentage of ^{55}Fe release was calculated according to the following equation: % ^{55}Fe release = [(cpm in supernatant)/(cpm in supernatant + cpm in cells)] × 100 (Ge et al., 2009).

2.6. Western blot analysis

Astrocytes received different treatments were washed with ice–cold PBS, homogenized with lysis buffer and then subjected to sonication using Soniprep 150 (MSE Scientific Instruments, London, UK). After centrifugation at 10,000*g* and at 4 °C for 15-min, the supernatant was collected, and protein content was determined using the Bradford assay kit (Bio-Rad). Aliquots of the cell extract containing 30 mg of protein were diluted in 2 ml sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% b-mercaptoethanol) and heated for 5-min at 95 °C before SDS–PAGE on 10% gel and subsequently transferred to a pure nitrocellulose membrane. After the transfer, the membrane was blocked with 5% blocking reagent in Tris-buffered saline containing 0.1% Tween-20 at 4 °C overnight. The membrane was rinsed in three changes of Tris-buffered saline/Tween-20, incubated in fresh washing buffer once for 15-min and twice for 5-min, and then incubated overnight at 4 °C with primary antibodies: mouse anti-rat TfR1 monoclonal antibody (1:1000); rabbit anti-rat DMT1+IRE, DMT1−IRE polyclonal antibodies and rabbit anti-mouse Fpn1 polyclonal antibody (1:5000). After three washes, the blots were incubated with goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibody (1:5000, Li-Cor) for 1-h at room temperature. The intensity of the specific bands was detected and analyzed by Odyssey infrared imagine system (Li-Cor). To ensure even loading of the samples, the same membrane was probed with rabbit anti-rat β-actin polyclonal antibody at a 1:2000 dilution (Ke et al., 2005).

2.7. Statistical analysis

Statistical analyses were performed using SPSS software for Windows (version 10.0) (SPSS, Inc., Chicago, IL). Data were presented as mean ± SEM. The difference between the means was determined by one-way ANOVA followed by a Student–Newman–Keuls test for multiple comparisons. A probability value